

AD _____

Award Number: DAMD17-01-1-0222

TITLE: Dynamics of Estrogen Receptor Transcription Complex
Assembly in Breast Cancer

PRINCIPAL INVESTIGATOR: Eli V. Hestermann, Ph.D.
Myles A. Brown, M.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040720 065

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2003	3. REPORT TYPE AND DATES COVERED Annual Summary (Jul 1, 2002 - Jun 30, 2003)	
4. TITLE AND SUBTITLE Dynamics of Estrogen Receptor Transcription Complex Assembly in Breast Cancer			5. FUNDING NUMBERS DAMD17-01-1-0222	
6. AUTHOR(S) Eli V. Hestermann, Ph.D. Myles A. Brown, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana-Farber Cancer Institute Boston, MA 02115 E-Mail: Eli_hestermann@dfci.harvard.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: ALL DTIC reproductions will be in black and white				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Estrogen plays a critical role in the development and progression of breast cancer. While endocrine therapies play an important part in breast cancer treatment, the failure of these therapies reflects a lack of knowledge concerning the molecular mechanisms involved in estrogen signaling. The biological activities of estrogen are mediated by estrogen receptors (ER). In addition, a large number of proteins termed cofactors are involved in ER signaling. Until recently, our knowledge regarding these cofactors was based on their ability to bind receptors <i>in vitro</i> and affect transcriptional activation in transfection experiments. The <i>in vivo</i> role of these cofactors and the specific target genes involved in breast cancer are not well known. Therapeutic agents, such as tamoxifen, also bind ER, but block proliferation in breast cells. However, tamoxifen increases the risk of endometrial cancer. We have used chromatin immunoprecipitation (ChIP) to investigate cofactor involvement in ER signaling <i>in vivo</i> and to understand the mechanisms underlying the different actions of tamoxifen in breast and endometrial cells. We are in the process of using ChIP to identify the set of genes regulated by ER and its cofactors in these tissues. The detailed understanding of tissue- and ligand-dependent changes in gene expression gained through these studies will lead to more effective therapies for ER-dependent breast cancer.				
14. SUBJECT TERMS Estrogen receptor, coactivators, gene cloning, gene transcription				15. NUMBER OF PAGES 16
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	9
Appendices.....	10

INTRODUCTION

Estrogen plays a critical role in the development and progression of breast cancer. While endocrine therapies play an important part in breast cancer treatment, the failure of these therapies reflects a lack of knowledge concerning the molecular mechanisms involved in estrogen signaling. The biological activities of estrogen are mediated by estrogen receptors (ER). In addition, a large number of proteins termed cofactors are involved in ER signaling. Until recently, our knowledge regarding these cofactors was based on their ability to bind receptors *in vitro* and affect transcriptional activation in transfection experiments. The *in vivo* role of these cofactors and the specific target genes involved in breast cancer are not well known. Therapeutic agents, such as tamoxifen, also bind ER, but block proliferation in breast cells. However, tamoxifen increases the risk of endometrial cancer. We have used chromatin immunoprecipitation (ChIP) to investigate cofactor involvement in ER signaling *in vivo* and to understand the mechanisms underlying the different actions of tamoxifen in breast and endometrial cells. We are in the process of using ChIP to identify the set of genes regulated by ER and its cofactors in these tissues. The detailed understanding of tissue- and ligand-dependent changes in gene expression gained through these studies will lead to more effective therapies for ER-dependent breast cancer.

BODY

Task #1 To identify the coactivators that are involved in the estrogen-induced transcription complex and to determine the sequence of events and the dynamics involved in the assembly and disassembly of the transcription complex

Status: Complete (see previous report)

Task #2 To compare the protein components of the tamoxifen-induced ER complex that occupies estrogen-responsive gene promoters in breast cancer cells and in endometrial cancer cells

- a) Identify coactivators/corepressors that participate in tamoxifen-induced ER complex formation at target gene promoters in breast cancer cells
- b) Identify coactivators/corepressors that participate in tamoxifen-induced ER complex formation at target gene promoters in endometrial cancer cells
- c) Define the sequence of events that are involved in the assembly and disassembly of tamoxifen-induced ER complexes in both breast and endometrial cancer cells

Status: Objectives (a) and (b) accomplished for a subset of genes (see previous report).

Objectives (a) and (b) are being pursued for additional ER targets. The novel RNA interference (RNAi) technique is being used to selectively knock down ER cofactors in order to determine gene-specific activity of those coregulator proteins.

The mechanisms of complex assembly and disassembly (objective c) are being pursued.

Task #3 To identify new estrogen- and tamoxifen-responsive genes in breast cancer cells and endometrial cancer cells

- a) Isolate DNA fragments by ChIP and identify ER-regulated genes using microarrays.
- b) Confirm the targets by quantitative RT-PCR of RNA from estrogen- and tamoxifen-treated breast and endometrial cells
- c) Apply ChIP using antibodies for coactivators and corepressors to identify the components of ER complexes at each gene
- d) Identify common and unique targets of ER in breast and endometrium

Status: The ChIP-microarray technique (objective a) has been developed and is beginning to produce data on genome-wide promoter binding by ER and associated cofactors (objective c). Expression microarray data has been mined to determine common and unique targets of ER in breast and endometrium (objective d), and targets of interest have been confirmed by quantitative RT-PCR (objective b).

Research this year has focused on developing the ChIP-microarray (CHIP²) technique that forms the centerpiece of Task 3. We are collaborating with Richard Young's group at the Whitehead Institute for Biomedical Research on this work. In parallel, gene expression microarray data have been used as a complimentary approach for identifying ER-regulated genes in breast and endometrial cells, and, in combination with RNAi, for determining the specific activities of individual cofactors. Target genes of interest have been validated, the role of specific cofactors in their regulation is being evaluated, and the genes' role in the proliferative response to ER ligands is being determined.

The CHIP² technique involves isolating factor-bound DNA by chromatin immunoprecipitation, amplifying that DNA by ligation-mediated PCR (LM-PCR), labeling the resulting DNA with fluorescent nucleotides, and hybridizing the sample to promoter microarrays (Ren et al, 2002). We have experienced difficulty in completing this technically demanding process, but have developed a protocol that produces adequate amounts of labeled DNA and are now identifying promoters specifically enriched in the ER-bound fraction versus total input DNA. We have refined the ChIP technique to achieve better enrichment of specific samples (currently ~50-fold, versus 20-fold last year) and have shown that this enrichment is maintained through the LM-PCR amplification (Figure 1A). Furthermore, the size distributions of the ER-bound and input fractions are identical and maintained through the LM-PCR (Figure 1B).

We have hybridized a mixture of labeled ER-bound DNA and input DNA to arrays with ~2000 promoters. Figure 2 shows that the ratio of signal from the IP and input (WCE) DNA is close to 1 for the vast majority of promoters, showing no specific binding of ER to those sequences. However, several promoters are specifically enriched in the ER fraction (10 at $p < 0.001$, 19 at $p < 0.01$). One barrier has been a lack of known ER target promoters for use as positive controls on these arrays. We are in the process of using the same techniques on a new generation of arrays in the Young lab, which have ~13,000 promoters, including most known ER target genes. Once estradiol-occupied ER targets have been identified on these arrays, we will begin looking at other ligands (such as tamoxifen) and other components of the activation complex (including cofactors such as the three p160 proteins, histone modifying enzymes, and components of the transcription machinery). We also plan to perform ChIP against specific histone modifications known to correlate with transcriptional activation (e.g. methylated lysine 4 or acetylated lysine 9 of histone H3) in treated and untreated breast and endometrial cells.

While the CHIP² technique was being perfected, expression profiling data produced in this lab and reported in the literature were mined for targets of ER ligands in breast and endometrium. We hypothesized that since tamoxifen causes proliferation in endometrial, but not breast cells, genes that are regulated by estradiol in both cell types but by tamoxifen only in endometrial cells would include the targets involved in

proliferation. Three hours of treatment with ligand was chosen to enrich for direct targets of ER, rather than downstream, secondary effects of treatment. While more than 100 genes were found to be upregulated by 3 hours of estradiol treatment in either MCF-7 breast cancer or ECC-1 endometrial cancer cells, only 20 of these targets were upregulated in both cell types (Figure 3). Furthermore, only two of these targets were upregulated by tamoxifen in ECC-1 cells. These genes, *fos* and *myb*, are both known oncogenes. Previous work in our lab (Shang and Brown, 2002) had indicated that the oncogene *myc* also filled these criteria, and a recent report (Hodges et al, 2003) indicated that cyclin D1 might also be a good candidate.

We used quantitative RT-PCR to follow expression of these targets over a longer time course (Figure 4). These data indicate that *fos* is induced by tamoxifen in MCF-7 cells at later time points, which removes it from consideration under our hypothesis. *Myc* does not appear to be induced by tamoxifen in ECC-1 cells, but because of its central role in cell proliferation, it is being included in future studies. We have designed RNAi oligonucleotides to knock down expression, and the optimal conditions for reducing expression have been determined (Figure 5). We are in the process of testing the ability of cells to proliferate in response to estrogen and tamoxifen following knock down of each of these targets. Regulation of critical targets will be investigated by ChIP.

In collaboration with other members of the lab, we have knocked down expression of each of the three p160 coactivators, SRC-1, TIF-2, and AIB-1, and assessed the effect of loss of these proteins on estrogen signaling (Figure 6). The data indicate that each of these cofactors acts with ER at unique promoters and in combination on others. We are repeating this experiment with tamoxifen treatment. We plan to combine these data with the results of cofactor CHIP² experiments to study the role of these cofactors in regulation of the full set of ER target genes.

KEY RESEARCH ACCOMPLISHMENTS

- The CHIP² protocol has been optimized for estrogen receptor and at known ER-responsive promoters and is being applied to discover novel targets
- Gene expression profiles have been mined to identify candidate genes involved in ER-mediated proliferation in breast and endometrium
- Regulation of candidate genes by estradiol and tamoxifen has been assessed in breast and endometrial cells
- RNAi has been optimized for these targets and is being used to assess their role in proliferative response
- RNAi is also being used to investigate the role of specific cofactors in ER-mediated gene regulation

REPORTABLE OUTCOMES

A manuscript detailing the identification of tissue-specific ER targets involved in proliferation has been submitted for publication.

Drs. Hestermann and Brown have presented this work at several conferences, including the American Association for Cancer Research and the Enzymology of Chromatin Keystone meeting.

Based in part on the success of this work, Dr. Hestermann was offered multiple faculty positions and will be leaving for Furman University this fall.

CONCLUSIONS

After experiencing difficulty in performing the CHIP² technique, we believe that we have the process working and are beginning to generate data on genome-wide promoter binding by ER and its associated cofactors. This will allow us to expand the work performed so far from a handful of target genes to the complete set of ER targets in breast and endometrium. In parallel to this work, gene expression data has been mined to investigate tissue- and ligand-specific gene regulation by ER and its cofactors. In addition, the ability to knock down expression of specific genes by RNAi has allowed us to determine the role of individual cofactors in ER-mediated gene regulation and to determine which targets are involved in proliferative responses to estradiol and

tamoxifen. These data will aid in the development of targeted treatments of breast and endometrial cancer that lack harmful side effects.

REFERENCES

- Hodges, L. C., Cook, J. D., Lobenhofer, E. K., Li, L., Bennett, L., Bushel, P. R., Aldaz, C. M., Afshari, C. A., and C. L. Walker. (2003) Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. *Mol. Cancer Res.* 1:300-311.
- Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A., and B. D. Dynlacht. (2002) E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints. *Genes Dev.* 16:245-256
- Shang, Y., Xiao, H. DiRenzo, J., Lazar, M. A., and M. A. Brown. (2000) Cofactor dynamics and sufficiency in estrogen receptor-mediated transcription. *Cell* 103:843-852.
- Shang, Y. and M. A. Brown. (2002) Molecular determinants for the tissue specificity of SERMs. *Science* 295:2465-2468.

APPENDIX COVER SHEET

FIGURES 1-6

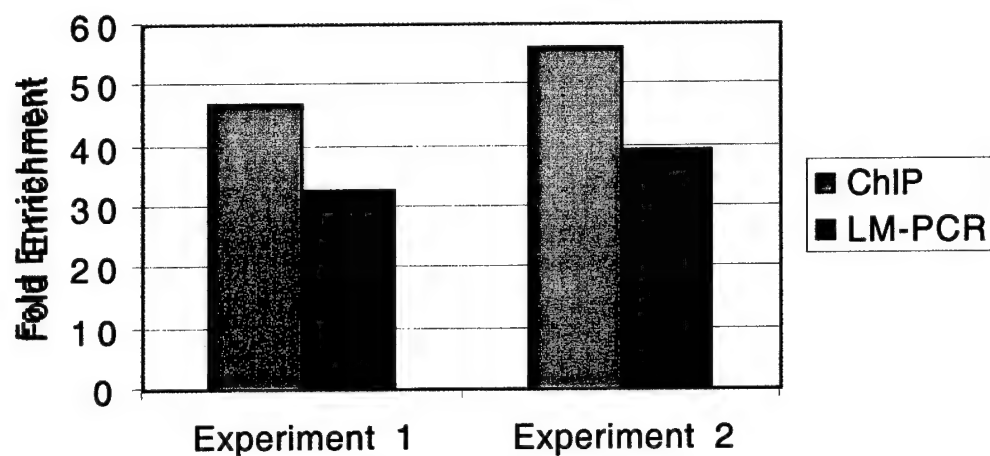
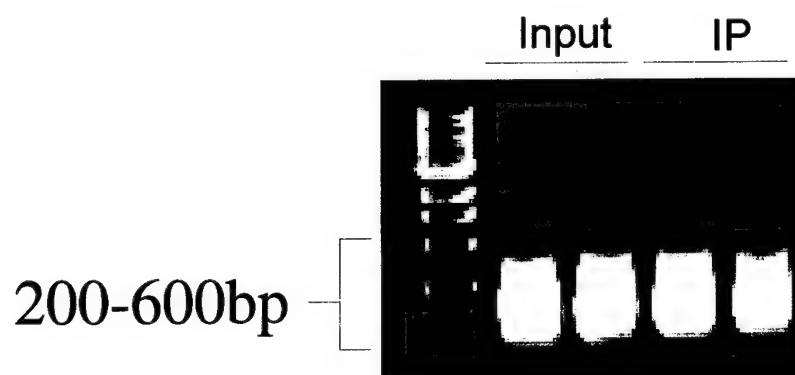
A**B**

Figure 1. Enrichment and fragment size of LM-PCR products for ChIP². A) The ER-binding region of the pS2 promoter was amplified by quantitative PCR from IP and input samples before (ChIP) and after (LM-PCR) LM-PCR, and the enrichment (relative to total DNA) in the IP fraction versus input is shown for independent experiments. B) Size of fragments following LM-PCR.

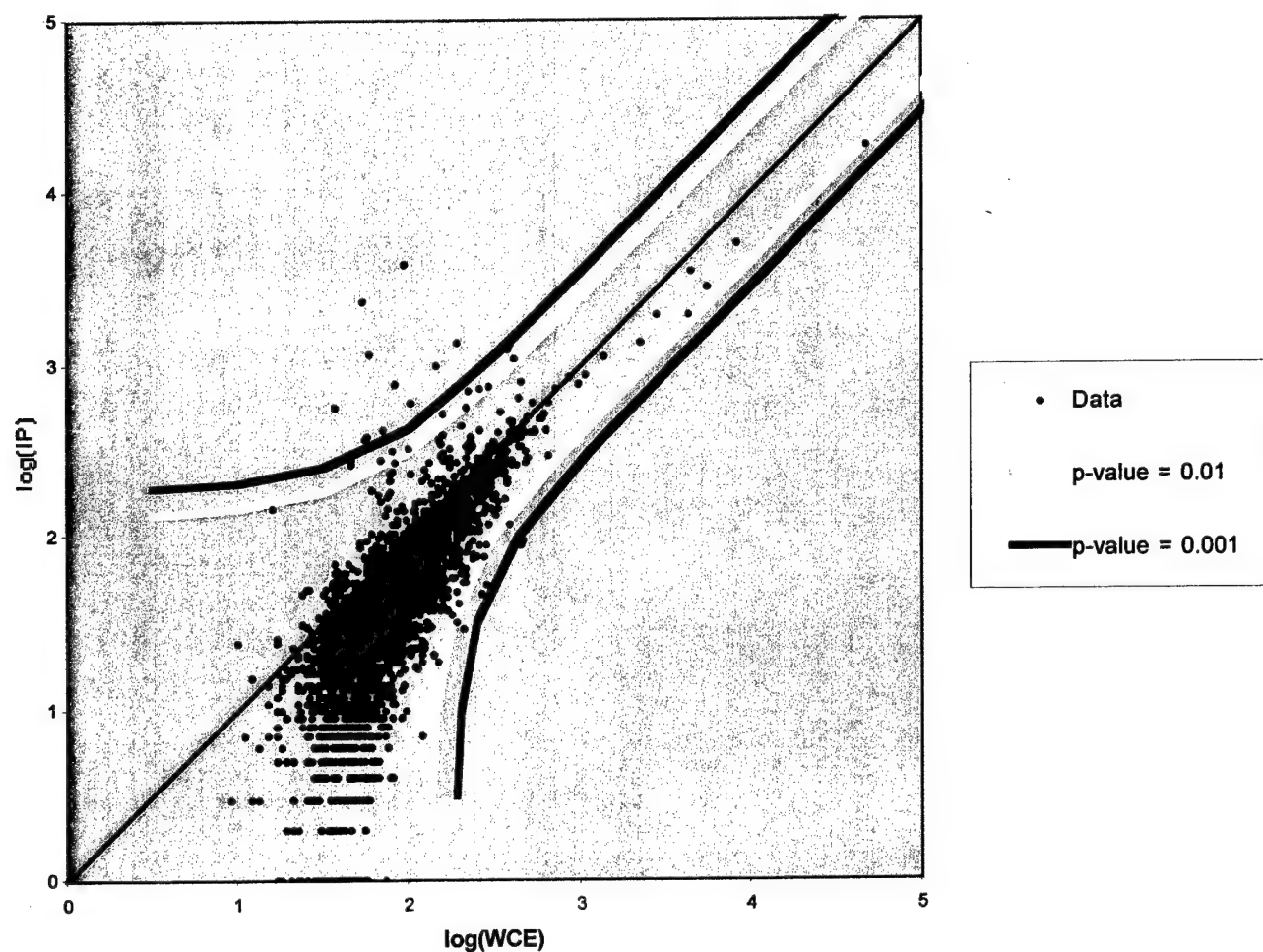


Figure 2. Signal from the ChIP² array. Log of intensity for signal from IP and input (WCE) channels is shown. Data points represent individual promoters spotted on the array.

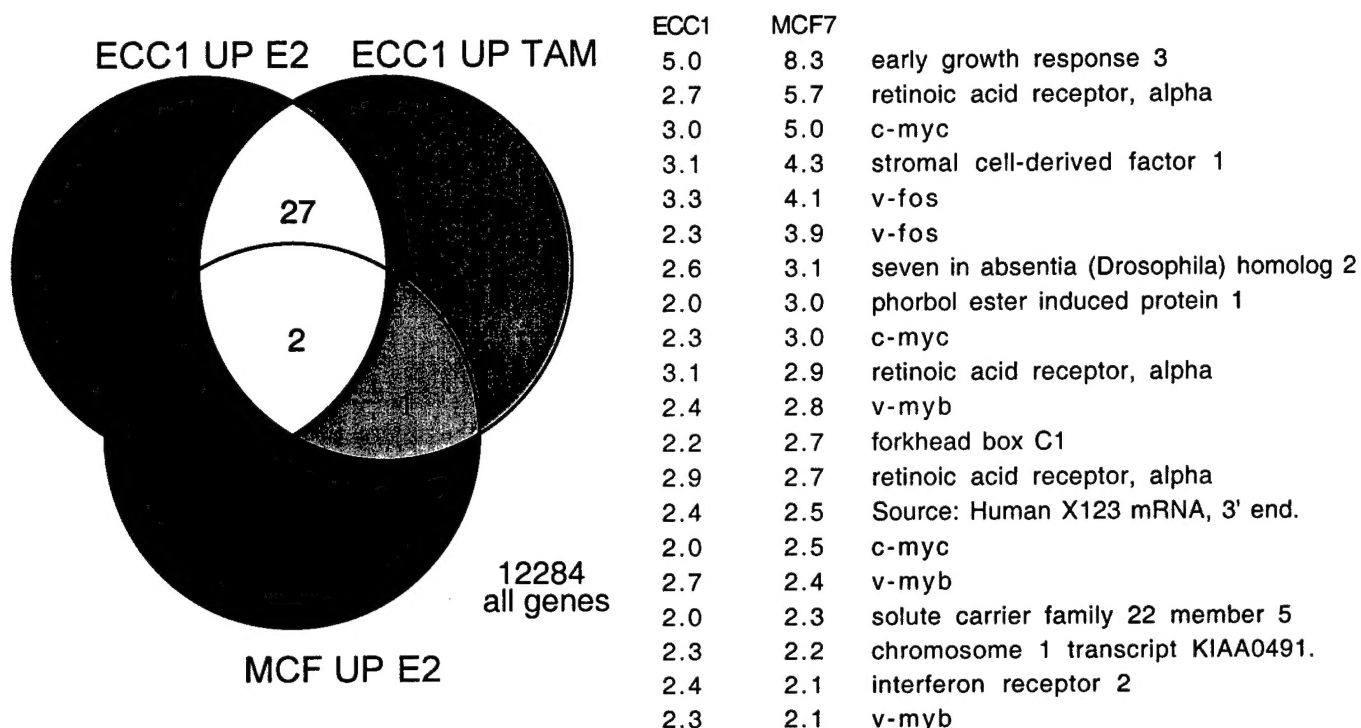


Figure 3. Genes upregulated by estradiol and tamoxifen in ECC-1, and estradiol in MCF-7, cells after 3 hours of treatment. Genes upregulated >2-fold were selected. The list shows fold increase in expression following estradiol treatment for genes upregulated in both cell types. Previously described ER targets are shown in red. Fos and myb are the two genes from this list that were also upregulated by tamoxifen in ECC-1.

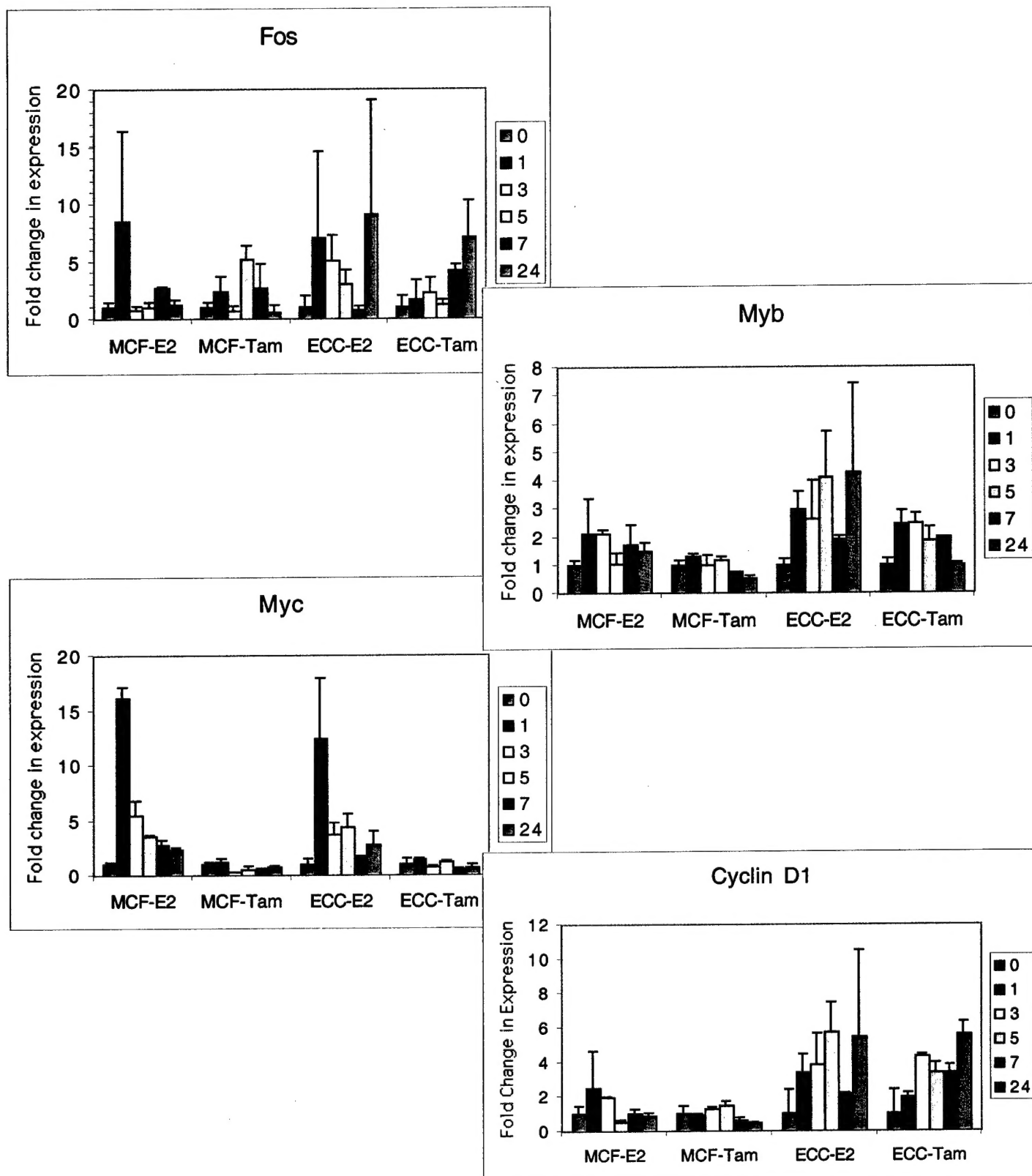


Figure 4. Expression of ER target genes in MCF-7 and ECC-1 cells following treatment with estradiol (E2) or tamoxifen (Tam) for the indicated number of hours. Expression was measured by quantitative RT-PCR and normalized to vehicle-treated cells.

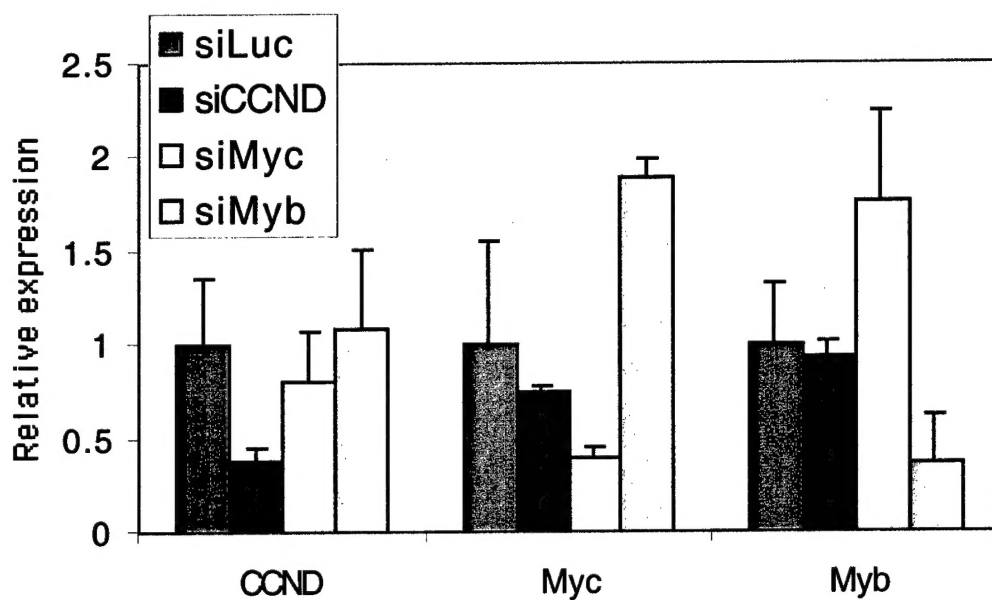
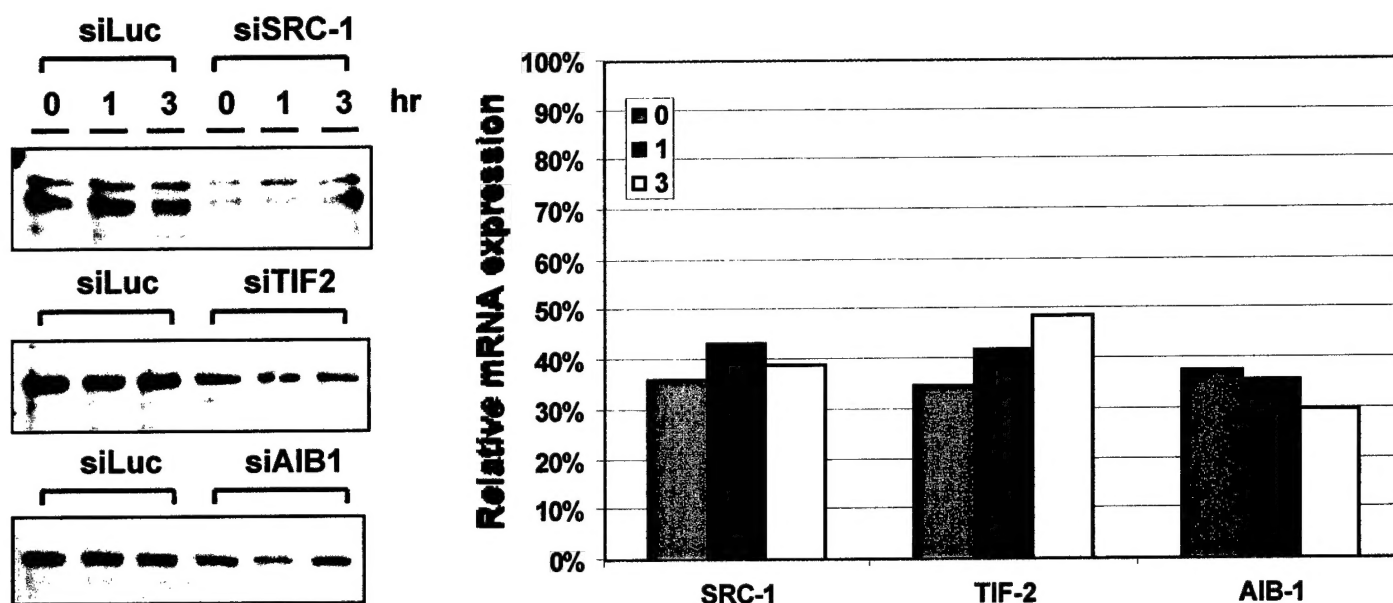


Figure 5. Expression of ER target genes in MCF-7 cells following RNAi treatment. Cells were transfected for two days with oligos to knock down expression of luciferase (negative control) or the indicated ER targets. Expression of RNA was measured by qRT-PCR.

A



B

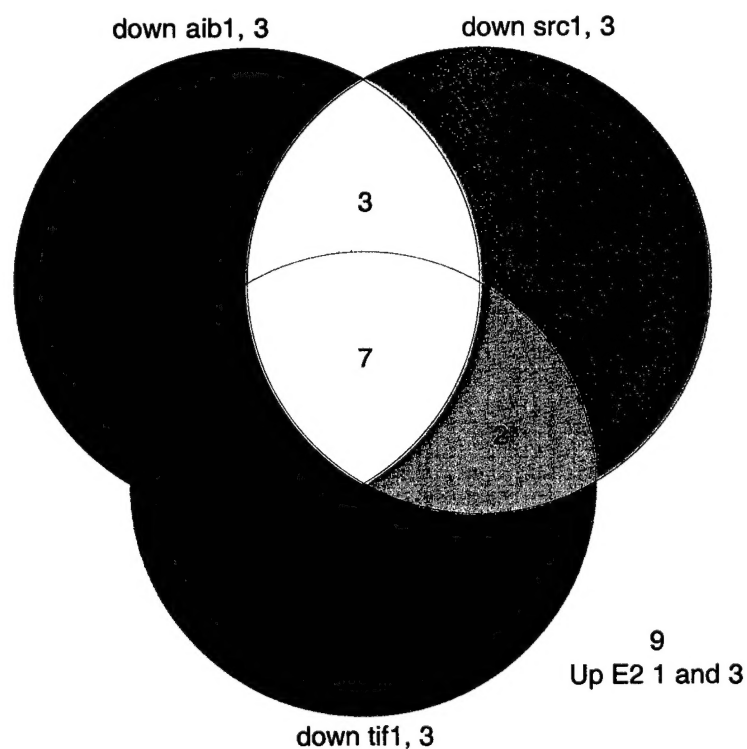


Figure 6. Role of p160 cofactors in ER-mediated gene regulation. Each of the three cofactors was specifically knocked down in MCF-7 cells, which were then treated with estradiol for 0, 1, or 3 hours. A) knockdown verified at both protein (immunoblot, left) and RNA (qRT-PCR, right) levels. B) Expression analysis was performed on RNA from treated cells. The number of genes showing lower expression in each knockdown treatment out of 36 genes upregulated by E2 at 1 and 3 hours is shown.